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High Molecular Weight Protein of Human Central Nervous System Myelin Inhibits Neurite Outgrowth: an Effect which can be Neutralized by the Monoclonal Antibody IN-1

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Abstract

Neurite outgrowth of PC12 cells in the presence of nerve growth factor and the spreading of 3T3 fibroblasts were inhibited by human myelin proteins from different areas of the central nervous system (CNS) in a dose-dependent manner. Application of liposomes containing human CNS myelin proteins induced rapid collapse of PC12 growth cones. When 3T3 fibroblasts were plated on a human CNS myelin protein-coated substrate the cells remained round, and spreading was inhibited. All these inhibitory effects could be neutralized by the monoclonal antibody IN-1, which was raised against a 250 kDa neurite growth-inhibiting protein (NI-250) of rat CNS myelin. Comparison of the inhibitory properties of human and bovine CNS myelin on PC12 neurite outgrowth showed that human CNS myelin was slightly more inhibitory per unit of myelin protein. Analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis revealed that in human myelin, as in rat and bovine myelin, a high molecular weight protein is responsible for the inhibitory activities on neurite outgrowth and fibroblast spreading.

Introduction

The regeneration of lesioned fibre tracts is very limited or absent in the mature central nervous system (CNS) of humans and higher vertebrates (Ramon y Cajal, 1928; Schwab and Bartholdi, 1996). However, transplantation experiments in which pieces of peripheral nerves or embryonic CNS tissue were grafted into the lesioned adult CNS showed the capacity for many of the CNS neurons to grow into the grafts, suggesting that, in principle, adult CNS neurons are able to regenerate (Tello, 1911; David and Aguayo, 1981; So and Aguayo, 1985; Tuszynski and Gage, 1992; Bregman *et al.*, 1993). Furthermore, in the myelin-free spinal cord (obtained by X-irradiation of newborn rats or by immunocytolysis) regeneration of some axons over long distances could be observed (Savio and Schwab, 1990; Keirstead *et al.*, 1992). These results, as well as *in vitro* studies (Caroni and Schwab, 1988a; Schwab and Caroni, 1988) indicate that oligodendrocytes and their product, CNS myelin, contain components with inhibitory properties for neurite growth and regeneration. Monoclonal antibodies (mAbs) were raised against a high molecular weight fraction of rat CNS myelin with potent neurite growth inhibitory activity. One of these antibodies, IN-1, was able to neutralize the neurite growth inhibition of CNS myelin (Caroni and Schwab, 1988b). *In vivo*, after lesioning the spinal cord of rats, mAb IN-1 induced long-distance regeneration of corticospinal tract fibres (Schnell and Schwab, 1990; Schnell *et al.*, 1994). Recently, pronounced behavioural improvements have been demonstrated in these mAb IN-1-treated, spinal cord-lesioned rats (Bregman *et al.*, 1993). These studies imply an essential role of the microenvironment, in particular of the inhibitory IN-1 antigens present in CNS myelin, in the ability of lesioned nerve fibres to regenerate in the adult brain and spinal cord.

In vitro, clear inhibition of adhesion and neurite outgrowth was shown for neuronal cells that were seeded onto cryostat sections of CNS tissue, especially on densely myelinated areas (Carbonetto *et al.*, 1987; Crutcher, 1989; Savio and Schwab, 1989; Watanabe and Murakami, 1990; Sagot *et al.*, 1991; Tutle and Matthew, 1991). These results are consistent with the potent growth cone collapse-inducing effects of cultured oligodendrocytes and CNS myelin in primary neuronal and neuroblastoma cells (Fawcett *et al.*, 1989; Bandtlow *et al.*, 1990). The mAb IN-1 was also able to neutralize the inhibitory properties of cultured oligodendrocytes and isolated CNS myelin *in vitro* (Caroni and Schwab, 1988b; Bandtlow *et al.*, 1990). Subsequent biochemical characterization indicated that, in rats, two membrane-bound proteins of molecular masses 35 and 250 kDa strongly inhibit 3T3 fibroblast spreading and neurite growth and cause the collapse of neuronal growth cones (Caroni and Schwab, 1988a; Bandtlow *et al.*, 1993; Igarashi *et al.*, 1993).

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The presence of negative signals in CNS myelin, resulting in the repulsion of growth cones and inhibition of the regrowth of neurites, has been found in many higher vertebrates, such as the opossum, cattle, and the chicken (Keirstead *et al.*, 1992; Rubin *et al.*, 1995; Varga *et al.*, 1995). Interestingly, the CNS myelin and oligodendrocytes of the adult *Xenopus* spinal cord (which do not show regenerative capacity), but not of the optic nerve (which is permissive to regeneration after axotomy), contain neurite growth inhibitors and stain positive with mAb IN-1 (Lang *et al.*, 1995). It was further shown for *Xenopus*, the opossum and cattle that the rat mAb IN-1 was able to neutralize the inhibitory properties of CNS myelin in various *in vitro* and *in vivo* assays (Lang *et al.*, 1995; Rubin *et al.*, 1995; Varga *et al.*, 1995).

For adult human CNS tissue, as for other higher vertebrates, non-permissive substrate properties for neurite growth are expected but so far have never been shown. Here we demonstrate that myelin from different areas of the human CNS is a non-permissive substrate for neurite outgrowth of PC12 cells and 3T3 fibroblast spreading. It also causes growth cone collapse. All three activities can be neutralized by the mAb IN-1. Further biochemical analysis indicated that a high molecular weight protein is responsible for these inhibitory effects.

Materials and methods

CNS myelin preparation

Human spinal cord, cerebellum, frontal lobe and brainstem were obtained 12 h post-mortem from a 71-year-old female who had no history of neurological disease and had died from a cardiovascular disorder. Bovine spinal cord was obtained from the local slaughterhouse. CNS myelin from both sources was prepared as described (Caroni and Schwab, 1988a). Briefly, following removal of the meninges, spinal cord tissue was homogenized on ice in 20 mM HEPES, pH 7.4, supplemented with 0.25 M sucrose and a protease blocker cocktail containing (final concentrations) 1 mM EDTA, 2.5 mM iodacetamide, 1 mM phenylmethylsulphononyl fluoride, 0.1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin A. CNS myelin was isolated by centrifugation (100 000 *g*, 14 h, 4°C) on a discontinuous sucrose density gradient and was collected at the interphase of 0.25 and 0.85 M sucrose. The myelin was subjected to hypo-osmotic shock by two washes with water (20 ml water per ml myelin), subsequently resuspended in 10 mM HEPES, pH 7.4 containing the protease blocker cocktail, and stored for up to 3 months at –70°C.

Preparation of myelin proteins

CNS myelin was centrifuged at 100 000 *g* for 1 h at 4°C and the pellet (1 volume) was homogenized in two volumes of extraction buffer [60 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate), 100 mM Tris–Cl, pH 8.0, 10 mM EDTA pH 8.0, protease blocker cocktail] and extracted for 10 min at 4°C on a rotary shaker. After pelleting the insoluble material (100 000 *g*, 1 h, 4°C) the clear extract could be stored at –20°C for up to 1 week. This extract contained most of the inhibitory activity of myelin. Activity could be further enriched by column chromatography on an anion exchange column (Rubin *et al.*, 1995). Inactive fractions were taken as controls.

Reconstitution of inhibitory proteins in liposomes

Liposomes containing CNS myelin proteins were prepared with a few modifications as described (Brunner *et al.*, 1978). Briefly, a 10-fold excess (wet wt) of phospholipids (phosphatidylcholine) in cholate buffer (2.5% cholate, 30 mM HEPES buffer, pH 7.4, with protease blocker cocktail) was added to 100 μl of extracted myelin proteins (1 mg/ml). The lipid–protein mixture was dialysed (cut-off pore of 5 kDa) against PBS for 24 h at 4°C, stored at 4°C, and used within 1 week. In order to increase the density of the liposome solution, 50 μl of 1% methocel in Hank's solution was added to the mixture prior to the assay.

Gel analysis of inhibitory proteins from CNS myelin

CHAPS-extracted human CNS myelin proteins were separated by electrophoresis with a sodium dodecyl sulphate (SDS) 6% (wt/vol) polyacrylamide gel under reducing conditions according to the method of Laemmli (1970). Gel regions between 0 and 100, 100 and 200, and 200 and 300 kDa were cut out using a coloured molecular weight marker (Rainbow™, Amersham) as a reference. Proteins were passively eluted overnight at 4°C from the gel stripes in the presence of the CHAPS extraction buffer. The eluted proteins were subsequently tested for inhibitory activity in the PC12 neurite outgrowth assay. Protein determination was carried out by the method of Bradford (1976).

3T3 spreading assay

CHAPS-extracted myelin proteins were coated on four-well culture dishes (well area corresponding to 1 cm^2; Greiner, Nürtingen, Germany) overnight at 4°C at different protein concentrations (20, 10 and 5 μg/well). Control dishes were coated with extraction buffer. After washing the dishes twice with Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution, 6000 3T3 fibroblasts (NIH 3T3, from American Type Culture Collection) in 70 μl Dulbecco's modified Eagle's medium (DMEM) (Gibco/BRL, Gaithersburg, MD) with 10% fetal calf serum (FCS) (Biological Industries, Kibbutz Beth Haemek, Israel), 100 U/ml penicillin and 0.5 mg/ml streptomycin (PS) (Gibco/BRL) were plated on each well. After 1 h incubation at 37°C the cultures were fixed in isotonic formaldehyde solution (3.7% formaldehyde, 10 mM HEPES buffer, pH 7.0, 5% sucrose). The number of inhibited, round cells was compared with the total number of cells in five randomly chosen areas of each well. Every experiment was performed in duplicate.

Monoclonal antibodies IN-1 (Caroni and Schwab, 1988b) and O1 (Sommer and Schachner, 1981) were produced by hybridoma cells grown in DMEM with 10% FCS and PS in tissue culture flasks. For the treatment with monoclonal antibodies IN-1 and O1, the CNS myelin coated dishes were washed twice with Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution followed by incubation with undiluted hybridoma supernatant (100 μl/cm^2; 1–10 μg antibody/ml) containing either antibody for 30 min at 37°C. After removing the solution, 6000 3T3 cells per cm^2 (in 100 μl medium containing 50% hybridoma supernatant) were plated on the dishes and incubated for 1 h at 37°C.

PC12 neurite outgrowth assay

The PC12 neurite outgrowth assay was performed as described (Rubin *et al.*, 1995). PC12 cells [the subclone that responds rapidly to nerve growth factor (NGF) and grows independently of laminin; Hempstead *et al.*, 1992] were grown in RPMI 1640 medium (Gibco/BRL) with 10% horse serum (Seralab, Sussex, UK), 5% FCS and PS and pretreated with 100 ng/ml NGF (Harlan Bioproducts, Indianapolis, IN) for 2 days prior to the outgrowth assay. CNS myelin-coated dishes were prepared as described above and 4000 PC12 cells either in 100% RPMI 1640 containing 100 ng/ml NGF or in 50% RPMI 1640 containing 200 ng/ml NGF and 50% hybridoma supernatant were plated on the dishes. After 24 h the cells were fixed in 3.7%

formaldehyde, 10 mM HEPES buffer, pH 7.0, with 5% sucrose. The percentage of PC12 cells with neurites longer than the diameter of the cell body compared with the total number of cells in five randomly chosen fields was determined. The assay was done in duplicate for each experiment; ~150 cells were evaluated per well (1 well = cm^2).

PC12 growth cone collapse assay

Four thousand PC12 cells were plated on uncoated culture dishes and grown in RPMI 1640 containing 100 ng/ml NGF until 60–80% of the cells had formed long neurites with growth cones (2 days). Cultures were then placed on a heated stage (37°C) and monitored through an Olympus inverted microscope (IMT-2). About ten continuously moving growth cones were selected for further observations and 80 µl of the liposome–methocel mixture containing ~3 µg myelin proteins was added to 2 ml medium. Growth cones were observed for ~60 min and photomicrographs were made every 2 min with a charge-coupled device (CCD) camera. After 1 h, collapsed and uncollapsed growth cones were counted.

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis of all data was performed according to the one-tailed, paired Student's t test.

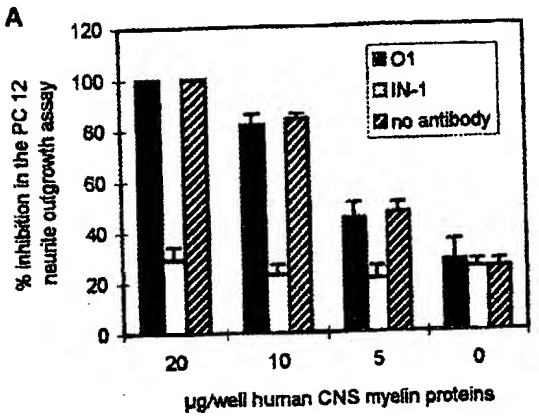
Results

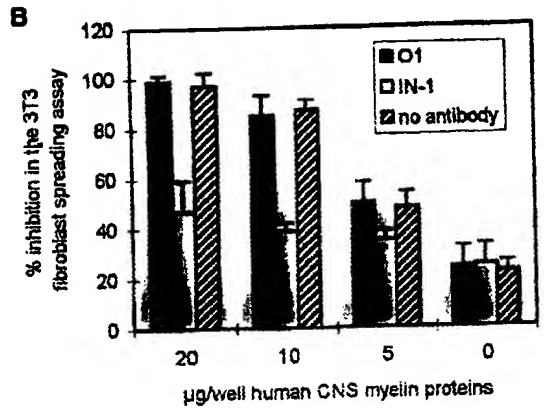
Human spinal cord myelin inhibits PC12 neurite outgrowth and 3T3 cell spreading, and causes collapse of PC12 growth cones

Culture dishes were incubated with different amounts of human spinal cord myelin proteins (20, 10, 5 µg), or with extraction buffer alone as a control. For antibody experiments the coated culture dishes were preincubated either with mAb IN-1, against the myelin-associated neurite growth inhibitors, or with mAb O1, which recognizes myelin-specific glycolipids (mainly galactocerebroside), as a control. PC12 cells or 3T3 fibroblasts were plated on the pretreated dishes. In the PC12 neurite outgrowth assay cells were attached to control substrate, and then 70 ± 5% (n = 8) of the cells formed neurites over 24 h. The presence of mAb IN-1 or control antibody O1 did not influence this result (Fig. 1A). Increasing amounts of human myelin proteins inhibited neurite outgrowth in a dose-dependent manner (Fig. 1A), leading to significant ($P < 0.01$) inhibition at 5 µg/cm^2 and complete inhibition of neurite outgrowth at 20 µg myelin proteins/cm^2. This inhibition of PC12 neurite outgrowth by human myelin could be very significantly ($P < 0.001$) decreased to control levels by mAb IN-1 (Fig. 1A).

About 75 ± 4% (n = 8) of the 3T3 cells were attached within 1 h and spread on the control substrate, leading to flat, round or polygonal fibroblast morphologies (Fig. 1B). Identical results were obtained in the presence of mAb O1 or IN-1. In contrast, on the human myelin-coated culture dishes 3T3 cell spreading was inhibited in a concentration-dependent manner (Fig. 1B): 50% of the cells were unable to spread on the wells absorbed with 5 µg/cm^2 myelin proteins ($P < 0.01$), and complete inhibition occurred on 20 µg/cm^2 human CNS myelin extract. In the presence of the mAb IN-1, however, the effect of CNS myelin was very significantly ($P < 0.001$) reduced and at least 50 ± 7% (n = 8) of the cells were able to spread on the highest concentration of CNS myelin. For both assays, treatment with mAb O1 had no effect (Fig. 1A, B).

Growth cone-collapsing activity of human myelin proteins was tested by the application of 20 µl liposomes containing ~1.5 µg/ml





C

Collapse response of PC12 growth cones to liposomes containing human CNS myelin proteins:

	growth cones observed	collapsed	% collapsed
hmyelin	21	17	81
hmyelin+ mAb O1	34	30	88
hmyelin+ mAb IN-1	32	14	44
inactive bmyelin	22	4	22

FIG. 1. Human CNS myelin inhibits PC12 neurite outgrowth (A) and the spreading of 3T3 fibroblast cells (B), and causes the collapse of PC12 growth cone (C). The effects of myelin are concentration-dependent and can be neutralized by the mAb IN-1, but not by the control antibody O1. PC12 cells (A) and 3T3 cells (B) were plated on culture dishes coated with different concentrations of human spinal cord myelin protein in the presence of mAb IN-1 or O1. Cells were quantified after 24 h (PC12 cells) or 1 h (3T3 cells) and values represent the mean ± SEM of four experiments. Liposomes containing ~3 µg human CNS myelin proteins were injected alone or together with IN-1 or O1 into PC12 growth cones. As a control, liposomes containing ~3 µg of inactive bovine myelin proteins were injected into PC12 growth cones (C). Collapsed growth cones were counted after 1 h.

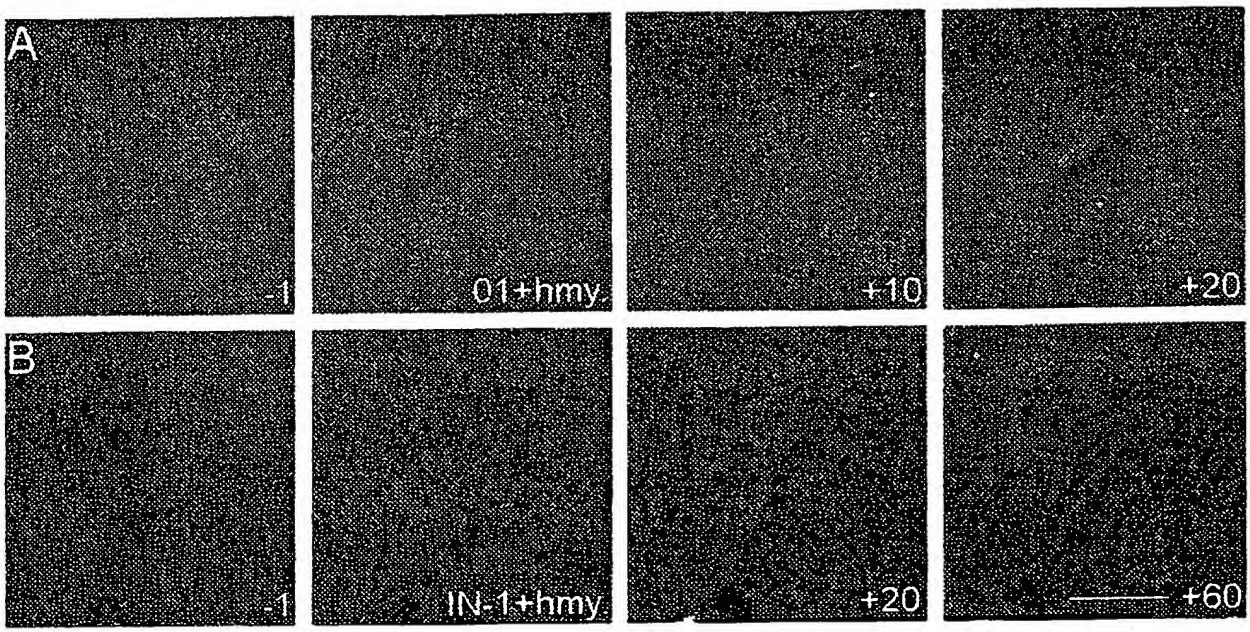


FIG. 2. Human CNS myelin proteins cause collapse of PC12 growth cones, an effect which can be prevented by co-injection of mAb IN-1. Liposomes containing ~3 μg human myelin proteins and either control antibody O1 (A) or IN-1 (B) were applied to PC12 growth cones. Pictures were taken 1 min before application, at application time, and (in A) 10 and 20 min or (in B) 20 and 60 min after application. Scale bar, 50 μm.

human myelin proteins to PC12 growth cones [the local area of the application corresponded to ~10% of the well area (0.1 cm^2)]. Eighty-one percent of the observed PC12 growth cones (n = 21) collapsed within 10–20 min (Figs 1C and 2A) and the neurites retracted within 30–60 min depending on the size of the growth cones (large growth cones seemed to be more resistant). Of the 22 observed growth cones, four (22%) collapsed in response to liposomes containing ~10 μg inactive bovine CNS myelin proteins (used as a control, obtained from an inactive anion exchange column fraction) (Fig. 1C). Application of mAb IN-1 together with the human myelin protein liposomes resulted in a 50% reduction of the collapsing effect of the myelin (Figs 1C and 2B), whereas the control antibody O1 had no effect (Fig. 1C).

These results demonstrate that human spinal cord myelin proteins have a strong inhibitory effect on PC12 neurite outgrowth and 3T3 fibroblast spreading, and cause the collapse of PC12 growth cones. The effects of human CNS myelin can be neutralized by mAb IN-1 raised against the rat neurite growth inhibitor NI-250, suggesting that this antibody cross-reacts with and neutralizes human inhibitory proteins present in the CNS myelin.

The specific inhibitory activity of human CNS myelin was significantly ($P > 0.01$) higher than that of bovine CNS myelin protein: 10 μg/cm^2 human and bovine CNS myelin proteins were compared in the PC12 neurite outgrowth and 3T3 spreading assay. Eighty-two percent (SEM 5%; n = 4) of the PC12 cells were inhibited on human myelin compared with 48 ± 3% (n = 4) on bovine myelin (data not shown). In the 3T3 spreading assay, 85 ± 10% (n = 4) were inhibited on human myelin and 66 ± 4% (n = 4) on bovine myelin (data not shown). The presence of mAb IN-1 neutralized the inhibitory effect of CNS myelin without any significant difference among the species (data not shown).

Inhibitory effect on PC12 neurite outgrowth is due to high molecular weight myelin proteins

Human spinal cord myelin proteins were separated by electrophoresis on a SDS 10% (wt/vol) polyacrylamide gel (Fig. 3). Proteins were eluted from the different molecular weight regions (0–100, 100–200, 200–300 kDa) and tested for inhibitory activity in the PC12 neurite outgrowth assay. Proteins with molecular weight <100 kDa or between 100 and 200 kDa did not show any detectable inhibitory activity (Fig. 3): 80% of the PC12 cells formed neurites and were unaffected by the presence of mAb IN-1 or the control antibody O1. In contrast, proteins with molecular weight between 200 and 300 kDa of human myelin were strongly inhibitory for PC12 cells: only 17 ± 11% of the PC12 cells formed neurites on these substrates. Monoclonal antibody IN-1 completely neutralized the inhibitory effects of these high molecular weight myelin proteins of human origin.

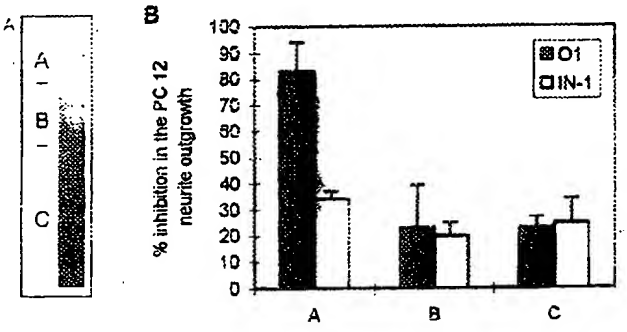


FIG. 3. Neurite growth inhibitory activity is associated with high molecular weight proteins of CNS myelin. Human spinal cord myelin proteins (extracted with CHAPS) were separated on 6% SDS-PAGE: gel regions (A, 200–300 kDa; B, 100–200 kDa; C, 0–100 kDa) were cut out (A) and the eluted proteins were coated on culture dishes. PC12 cells were plated, incubated for 24 h in the presence of either control mAb O1 or rat mAb IN-1 and evaluated. Values represent the mean ± SEM of three experiments.

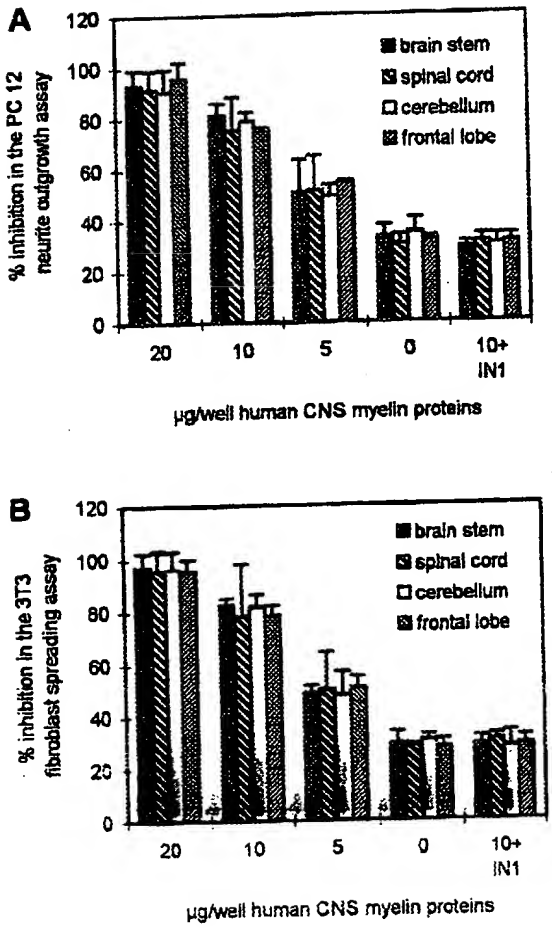


FIG. 4. Myelin proteins of human brainstem, spinal cord, cerebellum and frontal lobe have the same inhibitory effects on PC12 neurite outgrowth and 3T3 spreading. PC12 cells (A) and 3T3 fibroblasts (B) were plated on culture dishes coated with different concentrations (20, 10, 5, 0 µg/cm^2) of human CNS myelin from the different CNS regions. The neutralizing effect of mAb IN-1 was tested at a protein concentration of 10 µg/cm^2 for the chosen CNS regions. Cells with processes (PC12, 24 h) or spread cells (3T3, 1 h) were quantified. Values represent the mean ± SEM of three experiments.

Myelin from different regions of the human CNS has the same inhibitory effects on PC12 neurite outgrowth and on 3T3 cell spreading

The inhibitory activity of human myelin proteins from brainstem, spinal cord, cerebellum and frontal lobe was determined by the PC12 neurite outgrowth and 3T3 fibroblast assays. Different protein concentrations (20, 10, 5 µg/well) of myelin extracts from those regions or extraction buffer alone (control) were precoated on dishes. PC12 cells or 3T3 cells were plated on these substrates and within 24 h (PC12 cells) or 1 h (3T3 cells) the cultures were stopped and evaluated. The inhibitory activities on PC12 neurite outgrowth (Fig. 5A) and 3T3 spreading (Fig. 4B) were identical for myelin derived from brainstem, cerebellum, frontal lobe and spinal cord. Monoclonal antibody IN-1 was able to neutralize these inhibitory effects on neurite outgrowth and fibroblast spreading on each of the myelin extracts (data shown for 10 µg in Fig. 4A, B).

Discussion

Human CNS myelin is shown here to contain inhibitory proteins which prevent PC12 neurite outgrowth and 3T3 fibroblast spreading in a concentration-dependent manner. These inhibitory effects could be neutralized by the monoclonal antibody IN-1, which was raised against a 250 kDa fraction from rat CNS myelin (Caroni and Schwab, 1988b). Human myelin proteins also caused collapse of PC12 growth cones, which could be prevented by the application of mAb IN-1. The specific inhibitory activity of human myelin was slightly higher than that of bovine myelin. The inhibitory activities of human CNS myelin proteins were found in the high molecular weight range, between 200 and 300 kDa. Myelin from different CNS regions showed the same inhibitory effect on PC12 cells and 3T3 fibroblasts, suggesting that there were no major regional differences in the inhibitory substrate effect of myelin.

Earlier studies showed that rat CNS myelin contains two cell surface-associated proteins of 35 kDa (NI-35) and 250 kDa (NI-250), which are mainly responsible for the non-permissive substrate properties of rat oligodendrocytes and their product, CNS myelin, and for the collapse-inducing effect on neuronal growth cones (Caroni and Schwab; 1988a, b; Savio and Schwab, 1989; Bandtlow *et al.*, 1990; Bandtlow *et al.*, 1993; Igarashi *et al.*, 1993). A monoclonal antibody raised against NI-250 (mAb IN-1) could neutralize these inhibitory properties *in vitro* and allow regeneration of lesioned nerve fibres in the rat spinal cord and brain *in vivo* (Schnell and Schwab, 1990, 1993; Schnell *et al.*, 1994). IN-1 antigens are restricted to CNS myelin and are not detectable in peripheral nerves (Rubin *et al.*, 1994). Interestingly, the neurite growth inhibitory activity of myelin of the spinal cord of the opossum, *Xenopus* and cattle can be also neutralized by mAb IN-1 (Lang *et al.*, 1995; Varga *et al.*, 1995; A. A. Spillmann, C. E. Bandtlow and M. E. Schwab, unpublished).

The results of the present study show that human CNS myelin proteins exert significant inhibition of PC12 neurite outgrowth and 3T3 cell spreading at a concentration of 5 µg per cm^2 culture dish and totally inhibit the outgrowth and spreading at 20 µg per cm^2. Biochemical studies show that the active, inhibitory proteins in rat and bovine myelin represent a very minor part of the CHAPS-soluble myelin proteins (Caroni and Schwab, 1988a; Spillmann *et al.*, 1995). Human CNS myelin proteins also induced collapse of PC12 growth cones as well as growth cones of dorsal root ganglia (A. A. Spillmann, unpublished observations).

A comparison between bovine and human CNS myelin protein preparations demonstrated that human myelin was 20–40% more inhibitory than bovine myelin. After gel purification of the active components, this difference disappeared (A. A. Spillman, unpublished observations). This result suggests that human CNS myelin contains more inhibitory molecules per unit of white matter than bovine myelin.

Potent inhibitory activity could be found only in the molecular weight range between 200 and 300 kDa in human CNS myelin. Recent experiments showed that in bovine CNS myelin a single protein band of ~250 kDa correlated with strong collapse-inducing and neurite outgrowth-inhibitory effects (A. A. Spillmann, C. E. Bandtlow and M. E. Schwab, unpublished). in the rat, two components, of molecular weight 250 and 35 kDa, have been identified (Caroni and Schwab, 1988a). Both these components are inactivated by the mAb IN-1 (Caroni and Schwab, 1988b). In our experiments we found that the mAb IN-1 was able to fully neutralize human CNS myelin inhibitory activity in the assays for cell spreading, neurite outgrowth and growth cone collapse. In the absence of the full purification of these inhibitory constituent(s) of human myelin, it remains unclear whether one, several related or several different

molecules are involved. Although it has been shown that recombinant myelin-associated glycoprotein (MAG) had non-permissive substrate properties for adult dorsal root ganglion neurons, cerebellar neurons and the neuroblastoma cell line NG 108 (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994), recent studies suggest that MAG is not responsible for the inhibitory substrate effects on PC12 neurite outgrowth and 3T3 fibroblast spreading (Rubin *et al.*, 1995). No difference *in vivo* and *in vitro* could be observed in the inhibitory effects of CNS myelin and in the lack of regeneration when wild-type animals were compared with MAG gene knockout animals (Bartsch *et al.*, 1996). These results show that MAG seems not to be a major inhibitory component, at least in the mouse.

The neutralizing effect of mAb IN-1 on human CNS myelin suggests that IN-1 antigen(s) may contribute to the failure of axon regeneration following brain or spinal cord lesions also in humans. The observation that rat mAb IN-1 is able to neutralize the inhibitory effects of not only rat but also opossum, bovine, frog and human CNS myelin indicates a highly conserved IN-1 epitope among the different species.

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Abbreviations

CHAPS	(3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate)
CNS	central nervous system
FCS	fetal calf serum
mAb	monoclonal antibody
NGF	nerve growth factor
PC12 cells	rat phaeochromocytoma cell line
PS	penicillin + streptomycin
SDS	sodium dodecyl sulphate
3T3 cells	3T3 NIH fibroblast cell line

References

Bandtlow, C. E., Schmidt, M. F., Hassinger, T. D., Schwab, M. E. and Kater, S. B. (1993) Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. *Science*, 259, 80–83.

Bandtlow, C. E., Zachleder, T. and Schwab, M. E. (1990) Oligodendrocytes arrest neurite growth by contact inhibition. *J. Neurosci.*, 10, 3837–3848.

Bartsch, U., Bandtlow, C. E., Schnell, L., Bartsch, S., Spillmann, A. A., Rubin, B. P., Hillenbrand, R., Montag, D., Schwab, M. E. and Schachner, M. (1995) Lack of evidence that the myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. *Neuron*, 15, 1375–1381.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, 72, 248–254.

Bregman, B. S., Kunkel-Bagden, B., Reier, P. J., Dai, H. N., McAtee, M. and Gao, D. (1993) Recovery of function after spinal cord injury: mechanisms underlying transplant-mediated recovery of function differ after spinal cord injury in newborn and adult rats. *Exp. Neurol.*, 123, 3–16.

Brunner, J. H., Hauser, H. and Semenza, G. (1978) Single bilayer lipid–protein vesicles forms from phosphatidylcholine and small intestinal sucrase–isomaltase. *J. Biol. Chem.*, 253, 7538–7546.

Carbonetto, S., Evans, D. and Cochard, P. (1987) Nerve fiber growth in culture on tissue substrata from central and peripheral nervous system. *J. Neurosci.*, 7, 610–620.

Caroni, P. and Schwab, M. E. (1988a) Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J. Cell Biol.*, 106, 1281–1288.

Caroni, P. and Schwab, M. E. (1988b) Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron*, 1, 85–96.

Crutcher, K. A. (1989) Tissue sections from the mature rat brain and spinal cord as substrates for neurite outgrowth *in vitro*: extensive growth on gray matter but little growth on white matter. *Exp. Neurol.*, 104, 39–54.

David, S. and Aguayo, A. J. (1981) Axonal elongation into peripheral nervous system 'bridges' after central nervous system injury in adult rats. *Science*, 214, 931–933.

Fawcett, J. W., Rokos, J. and Bakst, I. (1989) Oligodendrocytes repel axons and cause axonal growth cone collapse. *J. Cell Sci.*, 92, 93–100.

Igarashi, M., Strittmatter, S. M., Vartanian, T. and Fishman, M. C. (1993) Mediation by G proteins of signals that cause collapse of growth cones. *Science*, 259, 77–79.

Hempstead, B. L., Rabin, S. J., Kaplan, L., Reid, S., Parada, L. F. and Kaplan, D. R. (1992) Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. *Neuron*, 9, 883–896.

Keirstead, H. S., Hasan, S. J., Muir, G. D. and Steeves, J. D. (1992) Suppression of the onset of myelination extends the permissive period for the functional repair of embryonic spinal cord. *Prod. Natl Acad. Sci. USA*, 89, 11664–11668.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680–685.

Lang, D. M., Rubin, B. P., Schwab, M. E. and Stuermer, C. A. O. (1995) CNS myelin oligodendrocytes of the *Xenopus* spinal cord—but not optic nerve—are nonpermissive for axon growth. *J. Neurosci.*, 15, 99–109.

McKerracher, L., David, S., Jackson, D. L., Kottis, V., Dunn, R. J. and Braun, P. E. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron*, 13, 805–811.

Mukhopadhyay, G., Doherty, P., Walsh, F. S., Crocker, P. R. and Filbin, M. Y. (1994) A novel role of myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron*, 13, 1–20.

Ramon y Cajal, S. (1928) *Degeneration and Regeneration of the Nervous System*. Hafner, New York.

Rubin, B. P., Dusart, I. and Schwab, M. E. (1994) A monoclonal antibody (IN-1) which neutralizes neurite growth inhibitory proteins in the rat CNS recognizes antigens localized in CNS myelin. *J. Neurocytol.*, 23, 209–217.

Rubin, B. P., Spillmann, A. A., Bandtlow, C. E., Hillenbrand, R., Keller, F. and Schwab, M. E. (1995) Inhibition of PC12 cell attachment and neurite outgrowth by detergent solubilized CNS myelin proteins. *Eur. J. Neurosci.*, 7, 2524–2529.

Sagot, Y., Swerts, J.-P. and Cochard, P. (1991) Changes in permissivity for neuronal attachment and neurite outgrowth of spinal cord gray and white matter during development: a study with the 'cryoculture' bioassay. *Brain Res.*, 543, 25–35.

Savio, T. and Schwab, M. E. (1989) Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. *J. Neurosci.*, 9, 1126–1133.

Savio, T. and Schwab, M. E. (1990) Lesioned corticospinal tract axons regenerate in myelin-free rat spinal cord. *Proc. Natl Acad. Sci. USA*, 87, 4130–4133.

Schnell, L. and Schwab, M. E. (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature*, 343, 269–272.

Schnell, L., Schneider, R., Kolbeck, R., Barde, Y. A. and Schwab, M. E. (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature*, 367, 170–172.

Schwab, M. E. and Bartholdi, D. (1996) Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol. Rev.*, 76, 319–369.

Schwab, M. E. and Caroni, P. (1988) Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading *in vitro*. *J. Neurosci.*, 8, 2381–2393.

So, K. F. and Aguayo, A. J. (1985) Lengthy regrowth of cut axons from ganglion cells after peripheral nerve transplantation into the retina of adult rats. *Brain Res.*, 328, 349–354.

Sommer, I. and Schachner, M. (1981) Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Dev. Biol.*, 83, 311–327.

Spillmann, A. A., Bandtlow, C. E., Keller, F. and Schwab, M. E. (1995) Purification of a neurite growth inhibitor from bovine CNS myelin. *Soc. Neurosci. Abstr.*, 612.5, 1560.

Tello, F. (1911) La influencia del neurotropismo en la regeneracion de los centros nerviosos. *Trab. Lab. Invest. Biol.*, 9, 123–159.

Tuszynski, M. H. and Gage, F. H. (1992) Bridging grafts and transient nerve growth factor infusions promote long-term central nervous system neuronal rescue and partial functional recovery. *Proc. Natl Acad. Sci. USA*, **92**, 4621–4625.

Tuttle, R. and Matthew, W. D. (1991) an *in vitro* bioassay for neurite growth using cryostat sections of nervous tissue as a substratum. *J. Neurosci. Methods*, **39**, 193–202.

Varga, Z. M., Schwab, M. E. and Nicholls, J. G. (1995) Myelin-associated neurite growth-inhibitory proteins and suppression of regeneration of immature mammalian spinal cord in culture. *Proc. Natl Acad. Sci. USA*, **92**, 10959–10963.

Watanabe, E. and Murakami, F. (1990) Cell attachment to and neurite outgrowth on tissue sections of developing, mature and lesioned brain: the role of inhibitory factor(s) in the CNS white matter. *Neurosci. Res.*, **8**, 83–99.